THE UNIVERSITY OF ALBERTA

THE EFFECT OF *ESCHERICHIA COLI* INFECTION ON HEALING SMALL BOWEL ANASTOMOSES IN THE RAT

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Effect of *Escherichia Coli* on Healing Small Bowel Anastomoses in the Rat" submitted by John P. Curry in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

This thesis describes work done in the Surgical-Medical Research Institute of The University of Alberta, Edmonton in 1968 and 1969 on the effect of bacterial infection on healing small bowel anastomoses in the rat.

A brief historical review is presented quoting early physicians and surgeons who pioneered present-day thinking in wound management.

In the introduction, the process of inflammation and fibroplasia is reviewed in considerable detail.

Next, the experiments carried out are described together with the results. The decreased polymorph leukocyte count at the wound site is regarded as an unexpected result.

In the discussion, theories are put forth in an effort to explain these unexpected findings.

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HISTORICAL REVIEW

Celsus, writing in the first century A.D., discussed the closure of abdominal stab wounds and stressed the need for anatomical closure. "Now stitching of the surface skin or of the inner membrane only is not enough but both must be stitched." (Zimmerman and Veith, 1967)

Theodoric lived in the 13th century and studied in the Bologna school under Hugh of Lucca. He wrote in 1267 of wounds anywhere in the body and also stressed the importance of accurate reconstruction: ". . .having brought the lips of the wound together, they should be replaced accurately in the position which they had in their natural state; if necessary, they should be held there by stitches taken in accordance with the size of the wound." (Zimmerman and Veith, 1967)

Jean Yperman, a native of Flanders, lived and worked in the latter part of the 13th century. He was a military surgeon and had considerable experience with wounds. He was aware of important surgical principles. He wrote concerning large wounds: "It is necessary to prick the suture points deeply enough so that the edges of the wound are approximated in the depths as well as the exterior, so that pus cannot accumulate in the depth, which occurs if only superficial edges of the wound are sutured and approximated, while the deeper parts are not." (Zimmerman and Veith, 1967)

Guy de Chauliac wrote *La Grande Chirurgie* in the first half of the 14th century. He has been described as the "Founder of Didactic Surgery." He discusses the treatment of wounds as follows:

"The common purpose in every solution of continuity, is union. This general and primary intention is accomplished by two (ways)

by nature as the principal operator, which works with its own powers, and with suitable nourishment; and by the physician as servants working with five intentions, which are subalternate, one to the other.

"The first requires the removal of foreign bodies, if there are any, between the divided parts.

"The second to reapproximate the separated parts, one to the other.

"The third, to maintain the reapposed parts in their proper form and to unite them as one.

"The fourth is to conserve and preserve the substance of the member.

"The fifth teaches the correction of complications." (Zimmerman and Veith, 1967)

Aureolus Phillipus Theophrastus Bombastus von Hohenheim, called Paracelsus, enjoyed a wide reputation in the first half of the 16th century. Paracelsus wrote on a wide variety of subjects with greater or lesser authority. He wrote on wound healing: "With every limb it should be understood that it carries it's own healing in itself, which heals it when it is wounded. Every surgeon therefore should know that it is not he who heals the wound but the balsam in the part that heals it. To be a good surgeon, you should protect and shield the natural balsam from ever present external enemies, so that they do not destroy the balsam nor poison or damage it but permit it to work and act under your protection." (Zimmerman and Veith, 1967)

Ambroise Pare (1510-1590) is said to have been the most celebrated surgeon of the Renaissance. He introduced many surgical practices but his surgical philosophy follows that of his predecessors and is quite simple: "I dressed him, and God healed him." (Zimmerman and Veith, 1967)

Matthaus Gottfried Purmann (1648-1771) was not the first surgeon to abandon the idea of "laudable pus." He was, however, one of the first to offer something to replace the compounds used to induce suppuration, to quote:

> "The Antients were of the Opinion that no Wounds could be cured without *Suppuration*; and directed their medicines accordingly. . . . I saw Reason enough to leave off this Method and adhere to a better, by the Invitation and Example of others, wherein I soon found it true, that wounds might be constantly and perfectly cured without *Suppuration* or the Application of *Plaisters, Balsams, Oyls, Ointments* and such like nastie greasie medicines. . . . Who knows not that *Nature* abhors Wounds or Divisions in any part of the admirable Fabrick of our tender Bodies? And therefore proposes and takes the readiest Way of conjoining and consolidating them; Nature knows that Wounds cannot endure being exposed to the *Air* because it irritates the *Nervous Fibers*, and so occasions *Inflammations* and Putrefactions and therefore contributes to the closing them up from that Enemy; and a Chirurgion being but Nature's Servant ought to do the like, with all possible Expedition; before any *Unctuous* and Oily Matter gives a check to it." (Zimmerman and Veith, 1967)

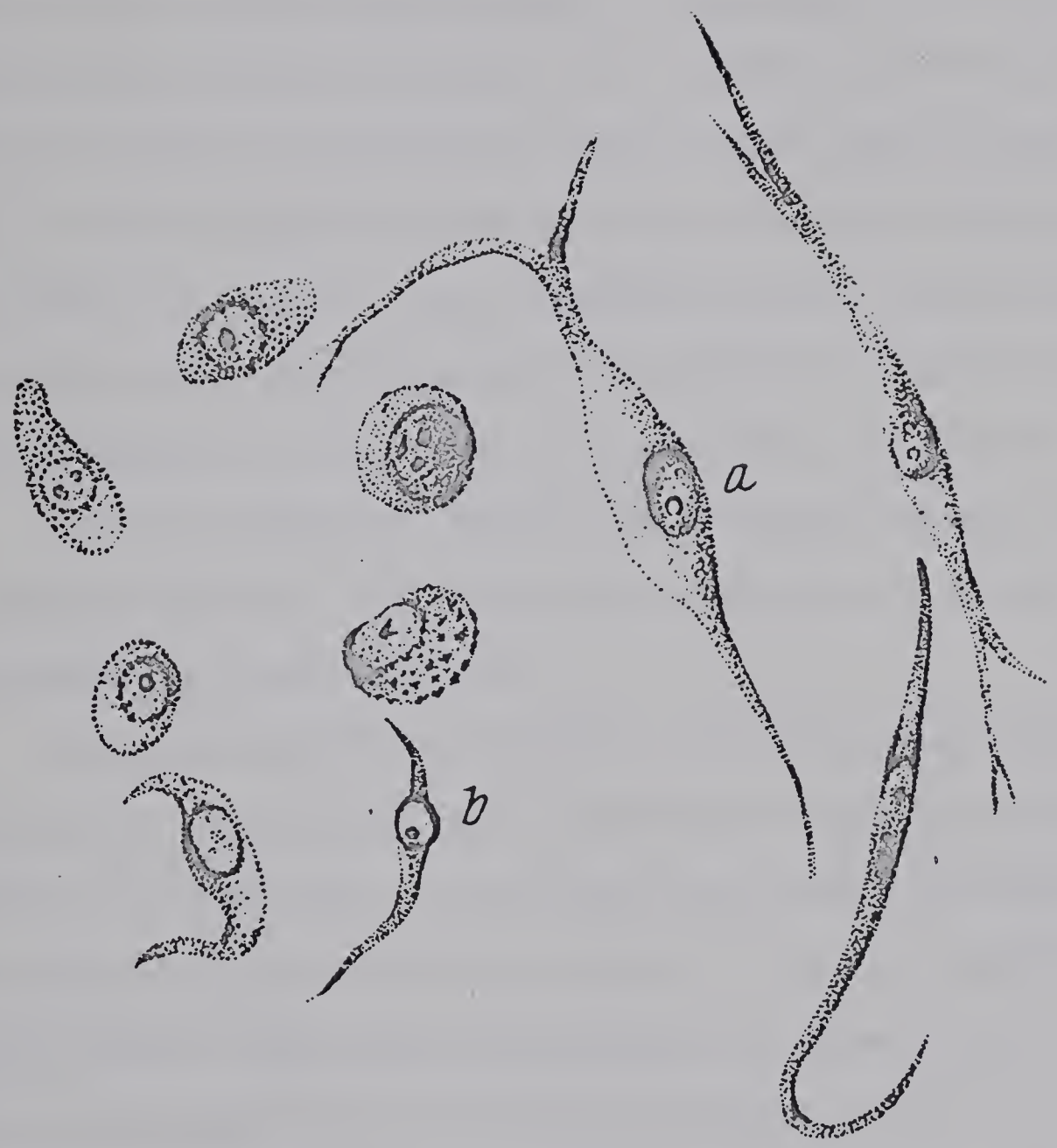
John Hunter (1728-1793) wrote: "A treatise on blood, inflammation and gunshot wounds." In this treatise he discussed the then current practise of enlarging all gunshot wounds. He felt that unless some structure was endangered by the ball, these wounds healed better and more quickly when left alone. It was Hunter's contention that a gunshot wound was essentially a clean wound and that any foreign matter or necrotic tissue would work its own way out as a slough. (Hunter, 1796)

In the middle and latter parts of the 19th century, the science of microscopic pathology gained more and more respectability. The fibroblast was described by Theodor Schwann for the first time in 1847 in the following words:

> ". . .they are represented as being spindle-shaped or longish corpuscles, which are thickest in the middle and gradually elongated in both extremities into minute figures. They may, therefore, be described as consisting of a thicker portion or body and fibres which proceed from it. The body is either round or slightly compressed upon the sides. The surface is covered with very minute granules. Within the thickest portion of it lies another small corpuscle of a circular or generally oval form and which again encloses one or two small dark points and accords entirely with the common cell nucleus. It is therefore probable that the entire corpuscle is a cell containing a nucleus." (Dunphy, 1963b)

There arose some controversy among the pathologists of the day as to whether these cells split up into fibres or whether they secreted fibres. Schwann, Remak and Schultze supported the former concept and Kolliker and Virchow the latter.

The exact connection between fibroblasts and the fibers of connective tissue awaited the work of Stearns in 1940.



The Fibroblast

INTRODUCTION

Wound Healing - Briefly

Originally wound healing was studied by taking sections at frequent intervals from healing tissue over a period of days or weeks, which were then studied histologically. Improvement in the methods of study came with the work of Clark, E. R. (1918) in studying the changes occurring in the transparent tail of the tadpole, following injury. Further improvement came with the invention by Sandison (1924) of the rabbit ear chamber. This transparent chamber actually permitted direct observation and photography of the microscopic changes occurring following injury, as well as those changes coming with healing.

While the rabbit ear chamber more accurately depicts conditions of a granulating wound, the lessons learned here can be applied to the wound healing by first intention.

Wound healing has been studied in many, many ways over the years. At a time when wounds could best be assessed by measuring the force required to pull them apart, it was found that wounds were weakest in the first four to five days. This was called, for lack of a better term, the "lag" phase. More recent investigation has shown that this early period of wound healing is, in fact, not a "lag" period -- it is a very active period and vital to the ultimate success of healing. The term "lag" has since been largely dropped to be replaced by the term "substrate". This is a more accurate term, connoting as it does the concept that a foundation is laid upon which healing proper can take place.

After the fifth day there is a rapid gain in strength of the

wound. This phase, which tapers off towards the end of the second week, has been variously called the "collagen", "fibroplastic", or proliferative phase. It is at this time when the fibroblasts which invaded the wound in the first five days actively secrete large amounts of collagen, lending strength to the wound.

The last phase in wound healing is one of remodelling and reconstruction. This phase is the longest and least spectacular of all. At this time the collagen "matures" by shrinking and in the process of remodelling gains strength while actually losing volume. This final phase of wound healing lasts for three to four months. At the end of this time the wound will have regained no more than 80% of the strength of the normal intact skin.

The substrate phase may be subdivided into three parts. The first twelve hours is the phase of acute inflammation. At this time the blood vessels in the region of the wound become more dilated, blood flow slows and localized thrombosis occurs. The wound space becomes filled with a cellular and fluid exudate. The cellular exudate is made up primarily of erythrocytes and polymorphs and in the second 24 hours, macrophages and fibroblasts become more prominent. The fluid exudate is made up of plasma and is rich in proteins. This exudate has a high osmotic pressure and tends to attract more fluid.

Overlapping the acute inflammatory response is the phase of demolition, which involves the destruction of injured cells and the removal of debris. Injured cells undergo autolysis following intracellular release of their enzymes. Invasion of polymorphs provides a source of enzymes which break down debris at the wound site. This may occur by destruction of the polymorph itself or by a process of phago-

cytosis by the polymorph. Polymorphs, in addition, have the ability to hydrolyze ground substance, permitting easier migration of cells. Monocytes, the true macrophages, are also part of the cellular exudate. These cells have the main purpose of engulfing debris which has been hydrolyzed by the enzymes of the autolyzed cells or the polymorphs. The macrophages are also believed to be an important link in the immune response. In addition to conveying information to the reticuloendothelial system, these cells may coalesce, forming multinucleate foreign body giant cells.

The third phase is that of granulation. Granulation tissue was originally a gross description given by surgeons to the tissue which invades an open wound approximately two to three days following injury. Because of the invasion of small blood vessels it has the appearance of multiple small, red granules in the wound. It consists, however, not only of blood vessels, but also fibroblasts. This phase overlaps with the latter parts of the first two phases. Fibroblasts migrate into the wound site along the fibrin strands of the clot. There has been considerable controversy over the source of fibroblasts but it is now felt that they arise from the loose perivascular connective tissue.

When the wound has been filled with capillary blood vessels and fibroblasts, it passes from the substrate phase into the proliferative phase. This takes place at about the third to fourth day. At this time the fibroblasts begin secreting large amounts of collagen. This phase lasts variably from five to ten days. The details of the biochemical formation of collagen and its molecular structure will be discussed in greater detail subsequently.

It should be mentioned at this time that in fact all three

phases may be going on in the same wound at the same time. In a healing ulcer, for example, there may be an area of acute inflammation at the centre which is surrounded by an area of demolition. Both these areas may be surrounded by an area of granulation.

The proliferative phase of collagen formation is followed by maturation. This stage of reorganization and remodelling lasts up to three months. At this time the great mass of collagen which has been set down contracts and is reorganized along lines of stress. At this time the blood vessels disappear and the scar changes from red to white.

Wound Healing - In Detail

A. Acute Inflammation - Morphology and Physiology

In this section I shall deal in rather more detail with the structure and function of the components of the healing process. In regards to the inflammatory response, this will involve discussion of neutrophils, macrophages, and new blood vessel formation. In the proliferative phase, discussion will centre around the fibroblast with a discussion of its role in collagen and mucopolysaccharide production.

1. Neutrophils

The workhorse of the cellular exudate in the early stages of acute inflammation is the polymorphonuclear (neutrophilic) leukocyte. This cell measures between 10-15μ in diameter. It has between 50 and 200 granules in its cytoplasm and a characteristic lobed nucleus. The leading edge of the cell has a ruffled border or pseudopod, while the trailing portion has a constriction.

The neutrophil passes through the stages of myeloid maturation, starting with the myeloblast and proceeding through promyelocyte,

myelocyte, metamyelocyte and juvenile to mature neutrophil. This growth cycle takes about three days (Bryant and Kelly, 1958). During this time the cell actively synthesizes protein (Pease, 1956; Low and Freemen, 1958). This is shown electron microscopically by a highly developed endoplasmic reticulum. As the cell matures, protein synthesis diminishes, and the number of granules increases. During the last two or three stages the cell nucleus develops its characteristic lobed appearance.

Neutrophils are currently believed to be "end cells". That is, they do not undergo any further mitosis. These cells, furthermore, can not be maintained in tissue culture for more than 72 hours (Osgood, 1954; Athens *et al.*, 1961; Brecher *et al.*, 1962).

There are between 20 and 30 billion neutrophils in the circulation of the normal man and an equal number sequestered or marginated. In addition, there are 50-100 cells in the bone marrow for every one circulating. The mechanism regulating maturation and release of neutrophils is unknown. The half-life of a neutrophil in the circulation is approximately six hours. Once it emigrates from the circulation, it does not return. These cells are excreted through the intestine, lung and skin. Those dying in the interstitial tissue are taken up by macrophages.

Neutrophils obtain their energy through the glycolytic pathway utilising glucose and producing lactic acid. Phagocytosis then can occur in a relatively oxygen-poor environment.

Neutrophil granules have been obtained by differential centrifugation. They have been found to lyse at a pH of five or less. These organelles contain several hydrolytic enzymes, all of which are active at an acid pH (Cohn and Hirsch, 1960). These enzymes include proteinases,

phosphatases, nucleases, nucleotidases, β-glucuronidase and arylsulphatase. In addition, neutrophil granules contain the antibacterial substances phagocytin and lysosyme (Hirsch, 1960a, b; Zeya and Spitznegel, 1963; Amano *et al.*, 1954; Repaske, 1956). Lysozyme is an aminopolysaccharidase which has the ability to degrade bacterial cell walls leading to lysis of the organism. Phagocytin is a protein whose exact structure and mode of action have not yet been elucidated. Very small concentrations of this substance have the ability to kill a wide range of bacteria without lysing them.

Neutrophils in an inflammatory situation become marginated. Why this occurs has been the subject of considerable study. One theory has been that the endothelial cells throw out cytoplasmic processes which "trap" the neutrophils (Williamson and Grisham, 1960). This phenomenon cannot be reproduced consistently. It is possible there may be electrochemical factors in the endothelial cell or on its surface which invite neutrophil emigration.

Neutrophil emigration has been explained by two theories. Benacerraf and others produced evidence that the endothelial cell is phagocytic. Marchesi (1962) on the other hand has shown using intravenous colloidal carbon that the primary site of egress is the junction between two endothelial cells. In this and other supporting work the basement membrane and the periendothelial sheath were shown to be more effective barriers than the endothelial cells themselves.

Once free of the vascular system, these cells move in an amoeboid fashion. *In vitro*, neutrophils move in a zig-zag manner at a rate of 35-40 μ/min. (Dittrich, 1962). The mechanism of this movement is not precisely known. There is no cytoplasmic streaming.

Neutrophils *in vitro* exhibit the property of chemotaxis (Harris, 1954, 1959). When these cells come within 100μ of a potential meal, random movement ceases and the neutrophil moves towards the particle. Numerous studies have been carried out in an attempt to elucidate the attractive agent which initiates chemotaxis. It has been stated that this phenomenon has never been demonstrated *in vivo*.

The process of phagocytosis is complex and has not been completely explained. Very small particles pass directly through the cell membrane of the neutrophil. Larger particles are engulfed by the cell. This process requires firm contact between cell and particle and also requires that the particle be covered by opsonins (Florey, 1962). For this reason, phagocytosis proceeds better in serum than in a protein-free solution. Phagocytosis does not require the presence of oxygen and proceeds well at an acid pH. When a particle has been completely engulfed by the neutrophil and the cell membrane has fused around it, a vacuole or "phagosome" is created (Weissman, 1965). The cytoplasmic granules lose their random distribution and encircle the phagosome. The granule membranes fuse with the phagosome membrane and discharge their enzyme contents into it. The vacuole created by the phagosome plus the granule is called a "phagolysosome" or more simply, a "digestive vacuole". In the digestive vacuole bacteria are digested. Proteins, lipids and nucleic acids are broken down. The granule enzymes are capable of degrading polymers, carbohydrates and lipids as well as phosphorylated or sulphated compounds (Sieracki, 1955; Valentine, 1955).

When digestion is complete and the vacuole contains only undigested material, the organelle is termed a "residual body". This residual body may remain as an intracytoplasmic structure or it may be

excreted into the extracellular environment (Wilson, 1953; Weissman, 1965).

Alternately, the cytoplasmic granules may rupture into the cell itself. This results in destruction of the neutrophil with consequent release of the granular enzymes into the inflamed environment. Weissman (1965) quotes Burke *et al.*, who have described a substance released from leukocytes which promotes vascular permeability.

2. Monocytes

Monocytes are the second of the two groups of cells making up the cellular exudate in the inflammatory response. These cells are the largest of the mononuclears, measuring 12-15μ across. They have more cytoplasm than lymphocytes, and when stained with Wright's stain, tiny azurophil granules become apparent. The nucleus of these cells is centrally located and stains lightly. A well-developed Golgi apparatus is apparent on electron microscopy, as is a smooth endoplasmic reticulum. Lysosomes are not present in monocytes (Cohn, 1965).

Macrophages are larger and more varied than monocytes, measuring between 15-80μ across (Maximow, 1932). Wright's stain, or Giemsa, shows a moderately basophilic cell, with a varying number of azurophil granules. The nucleus varies in size and shape and multi-nucleate forms are common. Vital staining shows a greater number of granules taking up the neutral red stain. Younger macrophages do not have as many granules as do the more mature cells. Granules tend to be localized to the perinuclear region as in the monocytes, and rosette formations are common. Phase contrast microscopy shows a constantly moving, undulating membrane surrounding the cell which is more highly developed than in many other cells. Small vacuoles arise in this membrane, which migrate into the

cytoplasm and disappear in the region of the centrosphere.

Macrophage ultrastructure differs considerably from cell to cell. A rough endoplasmic reticulum is often present, associated with ribosomes. These are segregated in the periphery and not associated with the Golgi complex. Granules may be scattered throughout the cytoplasm or they may be concentrated about the centrosphere. They tend to be bound by a single membrane but vary widely in size, shape, internal structure and electron density. Some granules have a homogeneous matrix while others are multi-vesiculated. Mitochondria are abundant and often lend a polarized appearance to the cell because of their close relation to the nucleus (Cohn, 1965).

Macrophages are found widely in the body and as a unit constitute the reticulo-endothelial system. Liver, spleen, lymph nodes, lungs and loose connective tissue are the commonest sites of macrophage concentration (Cohn, 1965). They are said to be as common as fibroblasts in this tissue (Maximow, 1902).

Macrophages arise from monocytes or by mitosis. *In vitro* studies have been done on suspensions of buffy coat cells. Initially, the cultures contain polymorphonuclear leukocytes, mononuclears and platelets. The polymorphs and lymphocytes degenerate early. The monocytes stick to the glass and phagocytose the degenerating cells. At two to three days following the start of incubation, the cultures show altered and larger monocytes with the development of a centrosphere adjacent to the nucleus. Neutral red granules and vacuoles are heterogeneous. At three to five days the monocytes are multinucleate and they may go on to form giant cells. Multinucleated forms occur because of nuclear replication without cytoplasmic separation (Cohn, 1965).

In vivo work done by Ebert and Florey (1939) using rabbit ear chambers, demonstrated that the transformation from monocyte to macrophage occurred in the tissues. Vital stains did not appear in macrophages until after the monocytes were extravascular.

There have been very few studies done on the energy metabolism of these cells. It would, however, appear from the available work (Borel *et al.*, 1959) that monocytes can utilise aerobic glycolysis more effectively than neutrophils, but in fact the major metabolic pathway is anaerobic with the production of lactic acid.

The cytoplasm of monocytes is rather unremarkable though it does contain more RNA than does that of neutrophils (Brucher, 1962). These cells also have a higher RNA:DNA ratio than do neutrophils. The synthesis of macromolecules by these cells may also be inferred from their extensive endoplasmic reticulum and ribosomes and well-developed Golgi complex (Cohn, 1965).

Direct analysis of blood monocytes for the presence of hydrolytic enzymes has not been carried out. Studies have been done, however, on alveolar and peritoneal macrophages and certain inferences may be drawn. These cells have been found to contain lysozyme, acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease, acid desoxyribonuclease, aryl sulphatase and lipase (Cohn and Weiner, 1963a). Alkaline phosphatase and peroxidase, two enzymes found in neutrophils and eosinophils, are absent in macrophages (Cohn and Hirsch, 1960; Archer and Hirsch, 1963). Cytochrome oxidase and succinic dehydrogenase are also found in macrophages in addition to the ones mentioned (Dannenberg *et al.*, 1963).

Studies on the fractionation of macrophages shows that these

cells are a particularly rich source of lysosomes. These findings appear to be corroborated by histochemical studies with staining for acid phosphatase. The staining corresponds to the membrane-bound vesicles which are apparent on electron microscopy (Novikoff, 1961).

Monocytes seem able to change their content of hydrolytic enzymes in response to a variety of stimuli. Increased amounts of acid phosphatase may be formed in the transformation of monocytes to macrophages (Weiss and Fawcett, 1959) or following a change of substrate in *in vitro* macrophage preparations (Suter and Hullinger, 1960). Injection of endotoxin brings about an increased amount of acid phosphatase production (Auzins and Rowley, 1962; Thorbecke *et al.*, 1961). The mechanism of this phenomenon is not understood.

In an inflammatory situation blood monocytes become part of the cellular exudate the same way neutrophils do: that is, by margination in the periphery of capillaries and emigration into the extravascular space. It is unlikely that there are subsequent "waves" of monocyte emigration (Clark *et al.*, 1936). Chemotaxis may have some bearing on the attraction of monocytes to the site of inflammation. This has been discussed previously. The work of Ebert and Florey in 1939 has shown conclusively that macrophages are derived from emigrated blood monocytes. The presence and accumulation of macrophages may follow any irritant. Lipids and waxes are particularly able to stimulate the accumulation of macrophages. These compounds are difficult for macrophages to digest and remnant debris may, in fact, alter cellular physiology (Ungar, 1955).

Phagocytosis by macrophages has been studied *in vivo* and *in vitro*. *In vitro* studies are usually carried out using suspensions of alveolar or peritoneal exudate macrophages. These cells are exposed to

bacteria and observed. *In vivo* studies are usually carried out indirectly by measuring the disappearance of injected particulate matter.

There are four factors which determine the rate and completeness of particle uptake (Cohn, 1965). (1) *serum factors* include a heat labile component (related to the complement system) and a heat stable component. Specific antibodies and opsonins fall into this latter group. (2) *Physical and chemical composition of the extracellular environment* includes pH, the supply of divalent cations and osmolarity. Surface phagocytosis is facilitated by rough surfaces or dense mesh networks, which trap particles. Phagocytosis is more easily accomplished on large particles than small, so (3) *particle size and aggregation* is a matter of some importance. (4) *Cell metabolism* is the fourth determinant of particle uptake (Cohn, 1962). It has been stated previously that macrophages derive their energy through both aerobic and anaerobic pathways. Blockade of the glycolytic pathway (by iodoacetate and fluoride) markedly reduces the rate of particle uptake. However, blockade of oxygen utilisation by cyanide or anaerobic conditions has no effect on phagocytosis.

Phagocytic activity may be stimulated or suppressed. Intravenous injection of lipopolysaccharide endotoxin tends to facilitate phagocytosis while reticulo-endothelial system blockade suppresses phagocytosis. This latter may occur because macrophages become "stuffed" or, which is more likely, opsonins which coat the particles are depleted (Cohn, 1965).

Phagocytosis (by peritoneal macrophages in the guinea pig) causes increases in oxygen consumption, glucose utilisation, lactic acid production, and incorporation of P^{32} into cell lipids (Oren *et al.*,

1963). It has been demonstrated that the enzyme content of granules decreases during phagocytosis while soluble enzymes increase (Cohn and Weiner, 1963b). Some enzymes leave the cell and this may be associated with their discharge into the phagosome. Macrophages are able to degrade erythrocytes and tissue cells. These cells also digest bacterial protein, nucleic acids and lipids at varying rates, lysozyme-sensitive organisms being digested first (Cohn, 1963a, b). Salmonella, Escherichia, Staphylococci and Streptococci are all killed within five to twenty minutes (Whitby and Rowley, 1959). The mechanism and bactericidal agents are different from those found in neutrophils (Hirsch, 1960).

Macrophages, in addition to possessing the abilities already mentioned, are capable of carrying out pinocytosis.* They also take up antigens, which may be altered by these cells. It is believed that information is then transferred to the immunologically competent cells (Cohn, 1965).

3. New Blood Vessel Formation

New blood vessels follow the macrophages into the inflammatory exudate. Schoefl and Majno in 1964 quoted the Clarks (1939) when they pointed out that "the same growth conditions which favoured the formation of new blood vessels also stimulated the growth of other tissues in the same region." No further comment on the stimulus to new blood vessel growth can be made at this time. New blood vessels grow at a rate of between 0.1 and 0.6 mm./day (Florey, 1962). Growth is favoured

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*Literally "cell-drinking". The ability of certain cells to surround, engulf and absorb droplets of liquid.

by balanced protein diets and elevated tissue histamine levels (Schoefl and Majno, 1964), and depressed by cool temperatures (Florey, 1962), cortisone treatment and histamine depletion (Schoefl and Majno, 1964).

Advancing blood vessels were seen under the electron microscope by Schoefl and Majno (1964). They studied the phenomenon using silver nitrate burns in rat corneas and small buttonhole wounds in the rat cremaster. These workers were in agreement with others who felt that new blood vessel formation cannot take place until after a clot has formed. The fibrin network seems to be required for cell migration. In the rat cornea blood vessels were seen advancing along loosened collagen lamellae. In the cremaster new capillaries developed in altered sarcolemma tubes.

All blood vessels start off as capillaries and later differentiate into either arterial or venous structures. Growth may take place in either loops or sprouts, depending on the severity of injury. A more severe injury stimulates the formation of solid sprouts, which shortly after formation become canalized. Milder injury stimulates the production of capillary loops.

The source of new blood vessels is the endothelial cell of existing blood vessels. These cells, when immature, have pseudopodia and migrate in amoeboid fashion. Under electron microscopy, young endothelial cells are seen to have an extensive endoplasmic reticulum and large mitochondria, indicating an active metabolic role. The large number of free ribosomes present is said to indicate more highly undifferential cells. Mature endothelial cells have many more vesicles than do immature cells, which indicates that they are more highly differentiated (Schoefl and Majno, 1964).

The basement membrane in new capillaries is either thin or incomplete, especially at the tips, and it becomes thicker as the vessel matures. Cells with a basement membrane have far less pseudopodia than those without one. Schoefl and Majno (1964) using colloidal carbon injected one hour prior to sacrifice demonstrated sites of leakage from newly formed blood vessels. They concluded that increased capillary permeability to plasma is the result of faults in the endothelial lining and to a lesser degree in the basement membrane. The basement membrane provides a barrier to intraluminal particles, and, when breached, these particles escape into the extraluminal space.

Mitoses in the endothelial cells of new blood vessels are seen proximal to the advancing front of cells. Only rarely do the migrating cells divide. The growth and canalisation of new blood vessels follows a very precarious course. They must grow sufficiently rapidly so that they do not burst from the pressure of flow of blood more proximally, but slowly enough that the blood within the lumen does not stagnate with resultant anoxia. New blood vessels are extremely fragile but their excessive permeability is a transient property, probably lasting no more than an hour (Schoefl and Majno, 1964). This permeability is probably the factor that prevents the vessel from bursting.

B. Proliferation - Fibroblasts

1. The Origin and Structure of Fibroblasts

In the study of wound healing, the most thoroughly investigated cell has been the fibroblast. This cell was first described by Schwann in 1847 (*vide supra*). It has always been associated with the formation of collagen. In addition, it is now believed to be the source for the mucopolysaccharide ground substance, which makes its appearance in the

first 24-36 hours following wounding.

Much study has been devoted to finding the source of the fibroblasts. Until recently there have been two theories concerning their origin. One group (Petrakis *et al.*) believe that the fibroblasts have a circulatory origin. This was supported by studies on buffy coat cells obtained by intracardiac punture and venipuncture. These studies demonstrated the differentiation of monocytes into fibroblasts in culture.

More convincing work by Grillo and Potsaid (1961) and Grillo (1963) using local radiation of wounds and autoradiographic tagging with tritiated thymidine supports the thesis that fibroblasts arise locally from a perivascular and loose connective tissue. This work involved radiating a wound 20 minutes after its creation. At this time the only cells with fibroplastic potential are those in the tissue at the time of wounding. These studies showed a 45% reduction in cell proliferation at the fifth day. Ross and Lilywhite (1965) repeated the work of Petrakis *et al.* and were unable to demonstrate collagen formation in their cultures. They concluded that the previous studies were marred by contamination with connective tissue cells.

Prior to the advent of electron microscopy, Stearns (1940) and others made careful drawings of fibroblasts and the way in which they developed fibres. The drawings show a spindle-shaped or stellate cell with a prominent nucleus. One group of cells appeared to be motile while a second group appeared to be actively synthesizing collagen. In Stearns' drawings numerous intracytoplasmic inclusions appear as clots. Electron microscopy has shown these inclusions to be made up of an extensive endoplasmic reticulum, a widely dispersed Golgi complex, mitochondria and occasional lipid droplets.

Ross (1968) summarized in greater detail the important features about this cell. He points out that the term "fibroblast" is applied to that cell which actively synthesizes collagen. On electron microscopy, one prominent feature of the fibroblast is its extensive rough endoplasmic reticulum. This structure is associated with cells that synthesize and secrete protein. The endoplasmic reticulum is distributed randomly throughout the cytoplasm and makes up approximately 35% of the volume of the cell.

Sites where the cisternae of the endoplasmic reticulum open onto the surface of the cell are commonly seen. It is probable that this is how the cell secretes its protein into the extracellular space.

The Golgi apparatus of the fibroblast is prominent and rather widely dispersed. It occupies approximately 9% of the cell's volume. In most secretory cells the Golgi complex bears a relatively fixed relation to the nucleus. However, these cells also tend to excrete their products from only one surface. The fibroblast, being in some cases a motile cell and having pseudopodia, can excrete from any surface. This is the probable explanation for the wide distribution of this Golgi complex.

The mitochondria are large and contain numerous cristae. The nucleus of the fibroblast is characteristically large and ovoid and usually contains one nucleolus.

2. The Structure and Synthesis of Collagen

Collagen is the protein synthesized by the fibroblast. It is widely distributed in mammalian tissue, occurring mainly in supportive structures. These structures include tendon, cartilage, bone and skin. It occurs in lower concentrations in intestinal walls, uterus and the

walls of blood vessels.

Collagen is rather an unusual protein containing virtually all the body stores of hydroxyproline and hydroxylysine. Stetten, in 1949, discovered that ingested hydroxyproline does not appear either in fibroblasts or newly formed collagen, whereas proline tagged with C^{14} appears in both places. Subsequent work has shown that the same holds true for lysine and hydroxylysine. The intracellular site of hydroxylation of these amino acids has not yet been determined. Collagen is approximately 12% hydroxyproline, 12% hydroxylysine and 30% glycine. Furthermore, it contains no cystine or tryptophane and very small amounts of tyrosine or methionine.

The basic unit of collagen is the tropocollagen macromolecule. This molecule has a molecular weight of approximately 300,000, a length of about 300 Å and a diameter of about 14 Å. The molecule consists of three polypeptide strands intertwined in a helix. Two of the three strands are believed to be identical (Ross, 1968). The strands are held together at lysine molecules by covalent aldehyde bonds. The collagen fibril is made up by aggregation of the tropocollagen macromolecules. These fibrils show periodic banding (under the electron microscope) between 600 and 700 Å apart. This banding is noticeable because the accumulation of amino acids at these points take up the stains.

The tropocollagen macromolecule is believed formed as are proteins in other cells. Free amino acids are picked up and react with adenosine triphosphate. Each amino acid has its own specific RNA and the "soluble" or "transfer" RNA for the amino acid in question, picks up the adenylated amino acid and transfers it to the ribosome on the rough endoplasmic reticulum. Many workers (Ross, 1968) have definitely

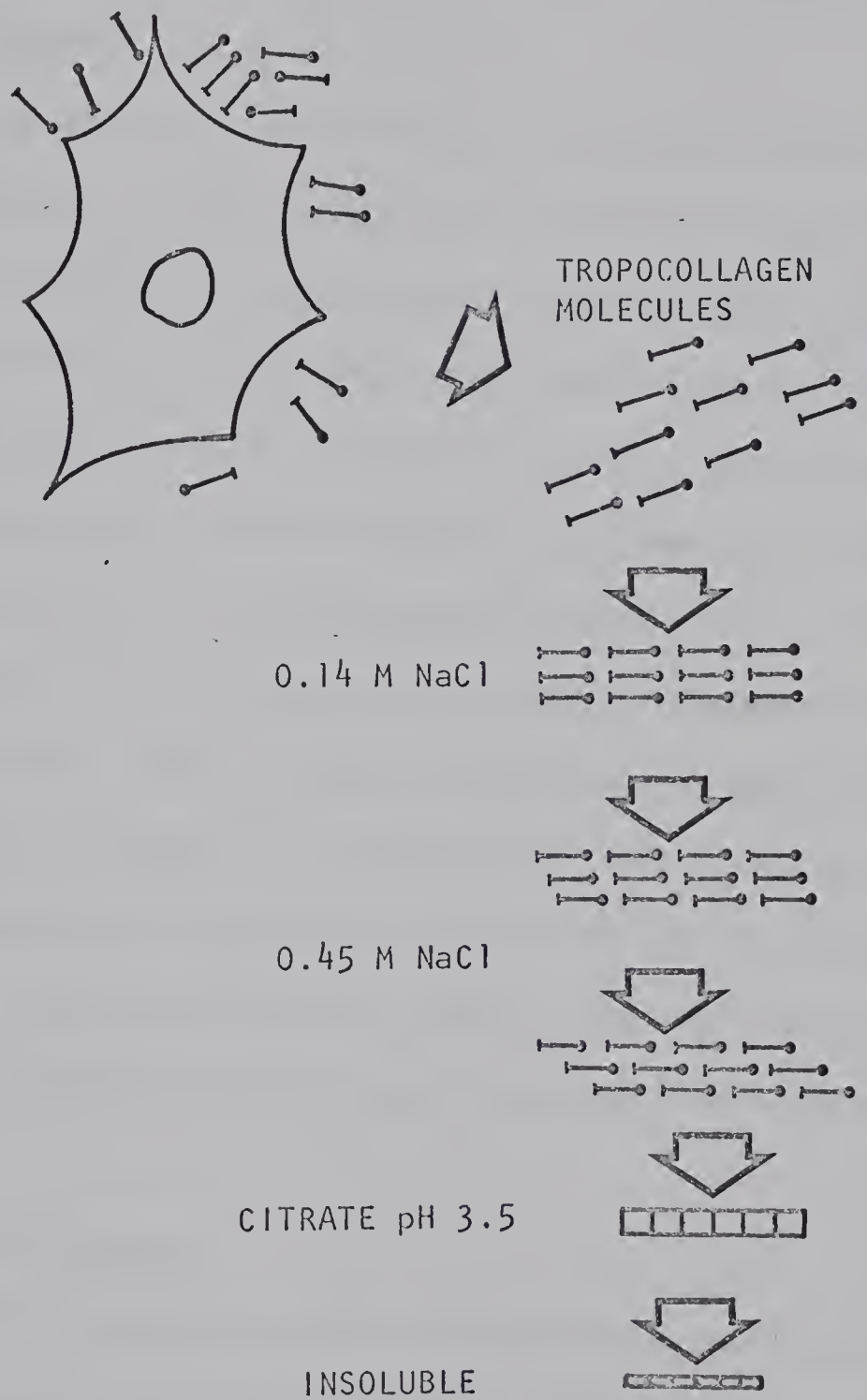
fixed the ribosome as the site of protein synthesis. The ribosomes contain messenger RNA, which have been coded on nuclear DNA. The soluble RNA-amino acid complexes align themselves along their opposite number, ribosomal (messenger) RNA. Hydroxylation of the lysine and proline residues does not occur until after the polypeptide has been synthesized. The site of the hydroxylation is believed to be the ribosome.

How the completed polypeptide strand is secreted from the fibroblast to become the tropocollagen macromolecule is not known. Two possibilities exist (Harkness, 1961). Synthesis of the three strands and aggregation into a macromolecule occurs within the fibroblast and further growth into a fibril awaits secretion. A second possibility having more support is that the aggregation of the three strands into the tropocollagen macromolecule takes place altogether outside the cell.

Once the macromolecules are secreted into the extracellular space, they aggregate into fibrils approximately 100 Å in diameter. After a length of time varying from a few days to a few months, a second population of fibres appears. These are thicker and more mature. They are believed formed by aggregation of the fibrils. The mechanisms governing the size and orientation of the fibrils is not understood.

Collagen has at least three solubilities and different amounts of a given volume of the protein are soluble in each different moiety. These variations in solubility have been clearly summarized in a diagram from Grillo (1964). The most recently formed collagen, tropocollagen, is easily soluble in cold physiologic saline. Hypertonic saline will dissolve fibrillar collagen, as will acid citrate. Old collagen may be completely insoluble. Acid citrate-soluble collagen was the first extracted (Nageotte, 1927 in Harkness, 1961). It was called "procollagen"

RELATIVE SOLUBILITIES OF COLLAGEN



(After Grillo, 1964)

by Orekhovitch and Shpikiter in 1958. Procollagen is the immediate forerunner of insoluble collagen and this stage may, in fact, be a very brief one (Harkness, 1961). The neutral salt-soluble collagen (those forms of the compound extractable with physiologic and hypertonic saline) are also present in the acid-soluble collagen if they have not previously been removed.

Collagen, once it passes through the various phases of solubility to become insoluble, is a relatively inert protein. Nevertheless, in some situations collagen is very rapidly degraded and/or absorbed. Healing fractures, healing granulation tissue and the postpartum uterus are examples. Work done by Gross and Lapiere in 1962, and Gross in 1964, brought forth the first identification of a collagenase. This compound was originally found in the metamorphosing tadpole tail. In wounds the inflammatory cells, mainly neutrophils, contain many hydrolytic enzymes including cathepsins. These enzymes are probably capable of degrading altered or "young" collagen at a wound site. That a certain amount of old insoluble collagen is degraded at the wound site is probable. How and how much are questions which are hard to answer. Furthermore, while a collagenase is probably present, both its nature and source remain unknown.

3. Mucopolysaccharides

In addition to collagen, the fibroblast has been shown to be the cell which synthesizes mucopolysaccharide. Mucopolysaccharide, the so-called ground substance, makes its appearance in the healing wound in the first 24-36 hours. Its source was formerly felt to be the mast cells. More recent work, however, has indicated that the mucopolysaccharides of significance in a healing wound are produced by the

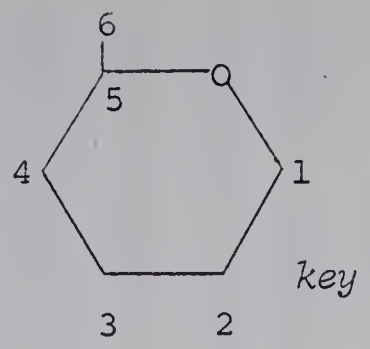
fibroblast.

The mucopolysaccharides of significance in association with collagen formation are six in number. They are: (1) heparatin sulphate (heparin monosulphate), (2) hyaluronic acid, (3) keratin sulphate, and (4), (5) and (6) chondroitin sulphates A, B and C. With the exception of the heparin compound which comes from mast cells, these glycoproteins are formed by the fibroblast.

Mucopolysaccharides, structurally are high molecular weight polymers, consisting of repeating units of A and B hexose rings. Generally one hexose ring is a uronic acid while the second is a sulphated amino sugar. Details are shown in the diagram from Harkness (1961).

Dunphy and Udupa (1955) correlated hexosamine levels in the healing wound with the amount of mucopolysaccharide present. They found that the hexosamine levels begin to rise immediately after wounding and peak on the third day, at the time when collagen is just beginning to be formed. As more collagen is formed (manifested by rising tensile strength and tissue hydroxyproline levels) the hexosamine level drops. The inference is that a high level of mucopolysaccharide is requisite to the formation of collagen, and as the amount of collagen increases, the amount of mucopolysaccharide decreases.

There is probably a considerable amount of truth in the foregoing statement. However, it must be remembered that, in fact, hexosamine levels reflect not only mucopolysaccharides formed at the site of the wound, but also glycoproteins from the serum. Boas in 1953 (Ross, 1968) showed that 50% of the hexosamine of rat connective tissue was due to extravascular serum proteins and probably unrelated to collagen synthesis. Bentley in 1967 (Ross, 1968) showed that less than



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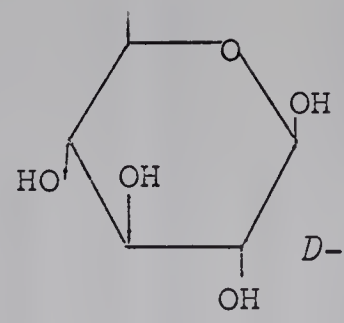
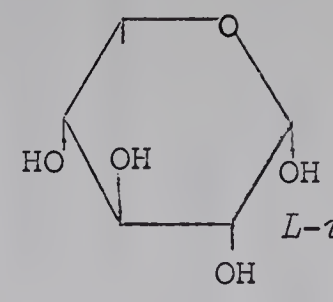
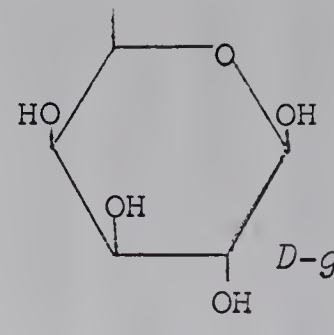


D-glucose



L-iduse



D-galactose

In uronic acids $-CH_2OH$ is replaced by COOH.

	Link A	Hexose I	Link B	Hexose II
Hyaluronic acid	(B) 1-4	D-glucuronic acid	(B) 1-3	D-glucosamine
Chondroitin sulphate A	B 1-4	D-glucuronic acid	(B) 1-3	D-galactosamine C_6 sulphate
Chondroitin sulphate C	B 1-4	D-glucuronic acid	(B) 1-3	D-galactosamine C_6 sulphate
Chondroitin sulphate B	B 1-4	L-iduronic acid	B 1-3	D-galactosamine C_6 sulphate
Keratosulphate	(B) 1-4	D-galactose	B 1-4	D-galactosamine C_6 sulphate

Numbers represent C atoms on each ring participating in link.

Hexosamines $-NH_2$ replaces -OH at C_2 and is acetylated.

( ) indicates probable B configurations.

(after Harkness, 1961)

20% of the hexosamine present in granulation tissue is in chondroitin sulphate and hyaluronic acid. The remainder exists in unidentified glycoproteins. It was shown by Gross (1957) that collagen fibrils can be formed *in vitro* in the absence of mucopolysaccharides. It has been believed by many workers that mucopolysaccharide provides some stabilization mechanism on which collagen formation can take place. Details of exactly how this works have not been worked out and are the subject of considerable investigation.

C. Factors Affecting Wound Healing

There are numerous factors which affect the rate and quality of wound healing. These factors may be divided into two groups, exogenous and endogenous.

The endogenous factors are those existent within the organism at the time of wounding. They include hypoproteinaemia, elevated levels of steroids, vitamin C deficiency, and the role of histamine.

Exogenous factors are those imposed on the wound from without and include bacterial infection and the technique of wound closure as it relates to intestinal anastomosis.

1. Endogenous

a. Proteins

Numerous workers from 1933 to the present have observed a decrease in tensile strength in wounds of protein-deficient animals. Dunphy and Udupa in 1955 noted that wounds in protein-deficient rats had a prolonged substrate phase with no collagen appearing until the seventh or eighth postoperative day. These workers found that the pattern of healing could be made to revert to normal by adding methionine to the diet. Other investigators have found that cystine has the same beneficial

effect on wounds. This is a most unexpected finding since collagen contains less than one per cent methionine and neither collagen nor mucopolysaccharide contain any cystine whatever. These findings would indicate that cystine and methionine have some supportive role in the various synthetic and secretory processes of the connective tissue cells (Ross, 1965).

b. Steroids

Steroid hormones are believed to affect wound healing (1) by their anti-inflammatory action, and (2) by a direct effect on the connective tissue forming cell. Numerous workers have shown that *in vivo* and *in vitro* cortisone and hydrocortisone cause decreased synthesis of connective tissue. Nocenti *et al.*, in 1964, studied the effect of cortisone and hydrocortisone on connective tissue formation on subcutaneously implanted polyvinyl sponges. They concluded that these steroids influenced the synthesis of protein but not collagen specifically.

It has been intimated that the primary effect of steroids is to depress the inflammatory response. Spain *et al.* (1952) gave cortisone and hydrocortisone 1 to 3 days prior to wounding. This treatment significantly decreased the tensile strength of healed skin wounds. The same steroids administered 2 days after wounding gave results indistinguishable from the controls. Savlov and Dunphy (1954) obtained similar results using Prednisolone.

Schiller and his associates have shown in two papers that steroids influence the synthesis of mucopolysaccharide. Schiller and Dorfman (1957) showed a decrease in the uptake of ^{35}S sulphate by chondroitin sulphate in animals treated with cortisone acetate. Schiller *et al.* (1965) found that hydrocortisone decreased the synthesis of both

chondroitin sulphate and hyaluronic acid.

Prednisolone had the effect of decreasing the synthesis of chondroitin sulphate while increasing the synthesis of hyaluronic acid.

Sandberg and Steinhardt (1964) suggested that cortisone administration decreased the amount of histidine decarboxylase released, thereby decreasing the amount of histamine in the wound. Histamine is felt to have a stimulatory role in collagen formation.

c. Histamine

Ross (1968) quotes several investigators who have studied the role of histamine in healing wounds in rat skin. These wounds have been shown to contain large amounts of histidine decarboxylase yielding high levels of tissue histamine, which has been correlated with increased tensile strength of the wound.

Sandberg in 1964 placed rats on a pyridoxine-deficient diet which tends to inhibit the formation of histamine. Wounds in these animals had decreased amounts of collagen in their granulation tissue. The wounds also contained less hydroxyproline and had a lower tensile strength than controls. Upon return of these animals to a normal diet, wound tensile strength increased.

d. Ascorbic Acid

Ascorbic acid has been known to influence the formation of connective tissue for centuries. Scurvy is a disease characterized by increased capillary fragility and destruction of scars and other connective tissue. Wolbach and Bessey in 1942 noted that collagen was either not produced or produced in a defective form in scurvy. Other investigators (Penny and Balfour, 1949; Persson, 1953) noted that scorbutic fibroblasts contained vacuoles. These were evident in specimens

prepared for routine histological examination.

In addition, it was noted that there was a substance present in the extracellular spaces in scurvy, which did not have the staining characteristics of collagen. Addition of ascorbic acid to the diet of these animals caused the formation of collagen in this substance in 24 hours.

Electron microscopy on the scorbutic fibroblast by several investigators (Ross and Benditt, 1962, 1964; Peach, 1962; Jorgenson, 1964) have shown several alterations in the cellular substructure.

(1) In the endoplasmic reticulum the cisternae become vacuolated and the aggregated ribosomes lose their characteristic configuration.

(2) There are an increased number of free ribosomes in the cytoplasm.

(3) Large numbers of lipid deposits appear in the cytoplasm.

(4) There is a lack of collagen in the extracellular spaces, though there are large mats of fibrinous-appearing material present.

Ross and Benditt (1964) noted that when ascorbic acid was given scorbutic animals, the fibroblasts showed evidence of return to normal within four hours (reappearance of ribosomal aggregates) and the cell was secreting normal collagen and indistinguishable from normal fibroblasts within 24 hours.

The nature of the extracellular material present in scurvy remains unclear. It was thought by many that the chemical defect in ascorbic acid deficiency was a failure of proline hydroxylation. This postulate makes probable the existence of a large pool of proline-rich collagen precursor. No evidence of such a pool has been found. It seems unlikely, therefore, that there is any conversion of an extracellular collagen precursor to collagen in the presence of ascorbic acid.

Whether hydroxylation of proline takes place before or after formation of peptide is also a question as yet unanswered. There is evidence to support both possibilities.

Gould in 1958 demonstrated that ascorbic acid has a local effect. Two polyvinyl sponges were planted subcutaneously in scorbutic guinea pigs. Ascorbic acid injected into one of the sponges stimulated the formation of collagen while none appeared in the other. Other work supported this finding.

2. Exogenous

a. Bacterial Infection

Escherichia coli is the commonest of the aerobic enteric bacteria. It is a gram-negative, motile rod present in greatest concentration around the ileo-caecal valve and becoming less concentrated both proximally and distally.

E. coli is a short organism, measuring from 1.0 to 4.0μ long by 0.4 to 0.7μ wide. It is peritrichous with a variable motility. It is aerobic and a facultative anaerobe. *E. coli* has three antigens: O, K and H. The H antigen is flagellar and not very pathogenic. The K antigen is capsular and covers the O, or somatic, antigen. The organisms are classified on the basis of their O and K antigens, their pathogenicity being a function of the latter two.

E. coli may cause three disease entities in man. Bladder and ascending urinary tract infections are most commonly caused by this organism. Certain especially virulent strains may cause severe epidemic diarrhea in infants, and thirdly, these organisms are the causative agents in non-epidemic "summer" diarrhea in two and three year old children. In addition to the foregoing, *E. coli* is frequently the organism

isolated from cases of appendicitis, peritonitis, endometritis and biliary tract infections.

Another aspect of the disease-causing potential of *E. coli* is that caused by its endotoxin. Endotoxin consists of the β-lipopolysaccharide component of the bacterial cell wall. This toxin is released only when the cell lyses after death (Smith, Conant *et al.*, 1960). Details on the local effects of endotoxin will be discussed more fully in a subsequent section.

Infection of wounds by bacteria generally has an unfavourable effect on healing. Work done by Hunt *et al.* (1967) demonstrated that connective tissue in the infected wound had the same weight as in the uninfected wound. However, the volume of tissue in the infected wound considerably exceeded that in the uninfected. The more voluminous tissue was more oedematous, fragile and more easily separated from its site of growth.

As a general rule it can be said that wound infection prolongs the inflammatory phase and delays healing. Fibroblasts cannot produce collagen in an infected bed. This subject will be dealt with in more detail subsequently.

b. Surgical Technique

The subject of end-to-end intestinal anastomosis has been widely investigated for over 80 years. It was Halstead in 1887 who first pointed out that the holding layer in intestinal anastomosis is the submucosa. Since that time the two-layer bowel anastomosis has been widely practiced. Usually this technique involves using a continuous suture of chromic catgut in the mucosa and submucosa. This may be followed by interrupted silk sutures in the seromuscular layer, for

reinforcement. Considerable work has been carried out recently (Ravitch *et al.*, 1967) assessing the differences between inverting and everting the mucosal cuff.

Fellows and co-workers in 1952, studied healing bowel anastomoses, using a variety of suture techniques. They subjected their anastomoses to bursting pressure studies. They found that sutures lent strength to the wound for only the first 3-4 days. Subsequently, wound strength was a function of collagen and/or fibrin rather than suture or suture material.

Beling (1957) published a clinical study in which he had carried out 60 single-layer anastomoses without a death. Letwin and Williams (1967) did extensive studies of intestinal anastomoses and comparing one- and two-layer techniques. The specimens were subjected to bursting pressure studies, as well as histological examination and biochemical assays for hexosamine and hydroxyproline. Their findings were as follows:

(1) Bursting pressures were consistently lower in animals with two-layer anastomoses than in animals with one-layer anastomoses. In animals which developed peritonitis, bursting pressures were lower than in animals without infection. Again, in the infected group, bursting pressures were lower in the two-layer group, as opposed to the one-layer group.

(2) Effective bowel lumen was decreased from 15-25% in the single-layer group throughout the test period, compared with a high of 50-60% in the double-layer group on the first two postoperative days.

(3) Histological examination revealed more granulation tissue in the one-layer group compared with the two-layer group. Furthermore, granulation tissue in the one-layer anastomosis was concentrated

between the healing edges compared with the two-layer group, where it was concentrated in the inverted cuff of mucosa and eventually sloughed out.

EXPERIMENTAL WORK

Introduction

Previous work in this Institute has been carried out investigating the effect of bacterial infection on the healing bowel anastomosis (Kilam, unpublished work). Results of this work indicated that sublethal infection around an intestinal anastomosis with a suspension of *E. coli* caused a significant decrease in the bursting strength of the anastomosis. Infection also decreased the number of polymorphs at the site of the anastomosis.

The purpose of the present work was to investigate this phenomenon further. Bursting strength determinations have been used in our work rather than breaking strength. In dealing with intestine, breaking strength determinations are rather an artificial and unphysiologic determination. No well-constructed bowel anastomosis is ever subjected to a linear force. For this reason we have followed the lead of Herrman *et al.* (1964), Nelson and Anders (1966) and Letwin and Williams (1967). Nelson and Anders (1966) have demonstrated that in bowel the law of Laplace is applicable. This states that the radial or transmural tension on the wall of a cylinder is directly related to the radius of the cylinder. In our work a single-layer anastomosis was constructed using interrupted sutures. These sutures were placed approximately 4mm apart and 2mm from the edge. This has two effects: first, it serves only to approximate the incised edges of the bowel. The suture line provides no real strength to a radial force directed at the anastomosis. Strength against a radial force in the one-layer anastomosis is provided only by the fibrin and granulation tissue between the

sutures. This is to be contrasted with a two-layer technique, using an inner continuous suture and an outer suture line constructed of interrupted sutures. In this situation, the inner circumferential suture lends strength against a radial force.

Secondly, the circumferential suture in the two-layer technique tends to narrow the bowel lumen, thus decreasing the radius at the site of the anastomosis. This means that the anastomotic site is, in fact, less likely to burst than it would be if a one-layer technique were employed, all other factors being equal.

Peripheral leukocyte counts were done pre- and postoperatively, and bone marrow smears were made at the time of sacrifice. These were done in an effort to determine whether or not there was any systemic reflection of the localized deficiency of neutrophils at the anastomosis.

Swabs of the anastomotic site were taken for culture at the time of sacrifice. This was done to verify that the organisms affecting the healing of the anastomosis were, in fact, the organisms which had been put in and not endogenous bacteria.

Materials and Methods

The experimental animals used were young male Sprague-Dawley rats weighing between 250-300 gms. The animals were individually housed, allowed water *ad lib.* and fed Purina Rat Chow. No animals were operated upon until a period of at least one week's observation had elapsed following their arrival in their quarters.

Prior to surgery the animals were fasted for 18 to 24 hours. They were permitted water during this time. This reduced intestinal contents to a very small quantity. Previous work had shown that animals

not so fasted were subject to postoperative bowel obstruction which markedly raised mortality. On the day of surgery, the animals were anaesthetized with an intraperitoneal injection of pentabarbital (4.0 mg/100 gm). A sample of peripheral blood was taken from the orbital sinus (Grice, 1964) for leukocyte counts.

The abdomen was shaved and prepped with an antiseptic iodine compound (Ioprep$^{(R)}$). A midline abdominal incision approximately 6.0 cm long was made and a loop of ileum brought out onto the abdominal wall. After the appropriate vessels had been ligated, a small (<1.0 cm long) piece of bowel was resected. A single-layer anastomosis was constructed, using seven to nine interrupted sutures of 6-0 black silk as before.

Following completion of the anastomosis it was checked for patency and the absence of gross leaks was verified. The bowel was then returned to the peritoneal cavity. In the experimental group 1.5 ml of a suspension of *E. coli* (#C59/69) (10^7 organisms/ml) was allowed to run over the anastomosis. In the control group, the anastomosis was not tampered with. The abdominal cavity was closed in two layers using 3-0 chromic catgut. The animals were not fed solid food until the morning following surgery. They were permitted water *ad lib*.

The animals were anaesthetized on each of the first five days postoperatively and the following studies carried out.

(1) Blood was taken from the orbital sinus for postoperative peripheral leukocyte count.

(2) On opening the abdomen and prior to removing the segment of bowel containing the anastomosis, a swab was taken for culture.

(3) The abdomen was examined for gross signs of infection.

(4) The segment of bowel containing the anastomosis was resected,

leaving a 3-5 cm cuff on either side of the anastomosis. Bursting pressure studies were carried out, using a mercury manometer connected to a reservoir of coloured water (Letwin and Williams, 1967). A 3 cm segment of bowel was connected to this device, the distal end occluded and the intralumenal pressure was increased at a constant rate of 10 mm Hg/sec. The point at which the anastomosis burst or obviously leaked was taken as the end point.

(5) Following bursting pressure studies, the specimens were fixed in 10% formalin and later stained with haematoxylin and eosin. Histological studies were carried out, using oil immersion and a Neubauer eyepiece reticle, and sections of 7μ thickness. The cells in the four outer quadrants of each of two fields were counted. The average number of cells per quadrant was obtained by dividing the total number of cells by 8, and the number recorded as cells per unit volume. Neutrophils, round cells, macrophages, and fibroblasts were counted.

(6) Bone marrow was obtained from the left femur. Smears were stained with Grunwald Giemsa. Two hundred cells were counted and differentiated between myeloid and erythroid precursors. E:M ratios were calculated.

At the conclusion of these studies the rats were sacrificed by exsanguination by cutting the inferior vena cava.

Results

A. Peripheral Leukocyte Counts

Pre- and postoperative leukocyte counts were compared. In both the infected and non-infected groups, the leukocyte counts postoperatively

were insignificantly lower than preoperatively for the first two days. For days 3 through 5, the postoperative counts were higher than before operation, again insignificantly (Table 1, Fig. 1).

B. Bone Marrow

The results of the cell counts and E:M ratio calculations are shown on Table 2 and Fig. 2. There was no significant difference between the two groups.

C. Bacteriology

Swabs taken from the anastomotic site at the time of sacrifice were cultured. These cultures all grew few to moderate relatively pure growths of the organism which had been introduced.

D. Pressure Studies

Bursting pressure studies showed significantly weaker anastomosis in the infected group than in the control group (Table 3, Fig. 3).

E. Histology

The histological studies of the anastomoses confirmed our earlier results. In the control group on days 1 and 2, the neutrophil levels were 665 and 472, falling to 290 by day 3 (Table 4, Fig. 4). The count fell to 151 on the fourth day and to 130 on the fifth day. This is contrasted with the infected group in which the level of neutrophils was highest on day 1 at 294 (of day 3 above) and subsequently fell with one upward rise on day 3.

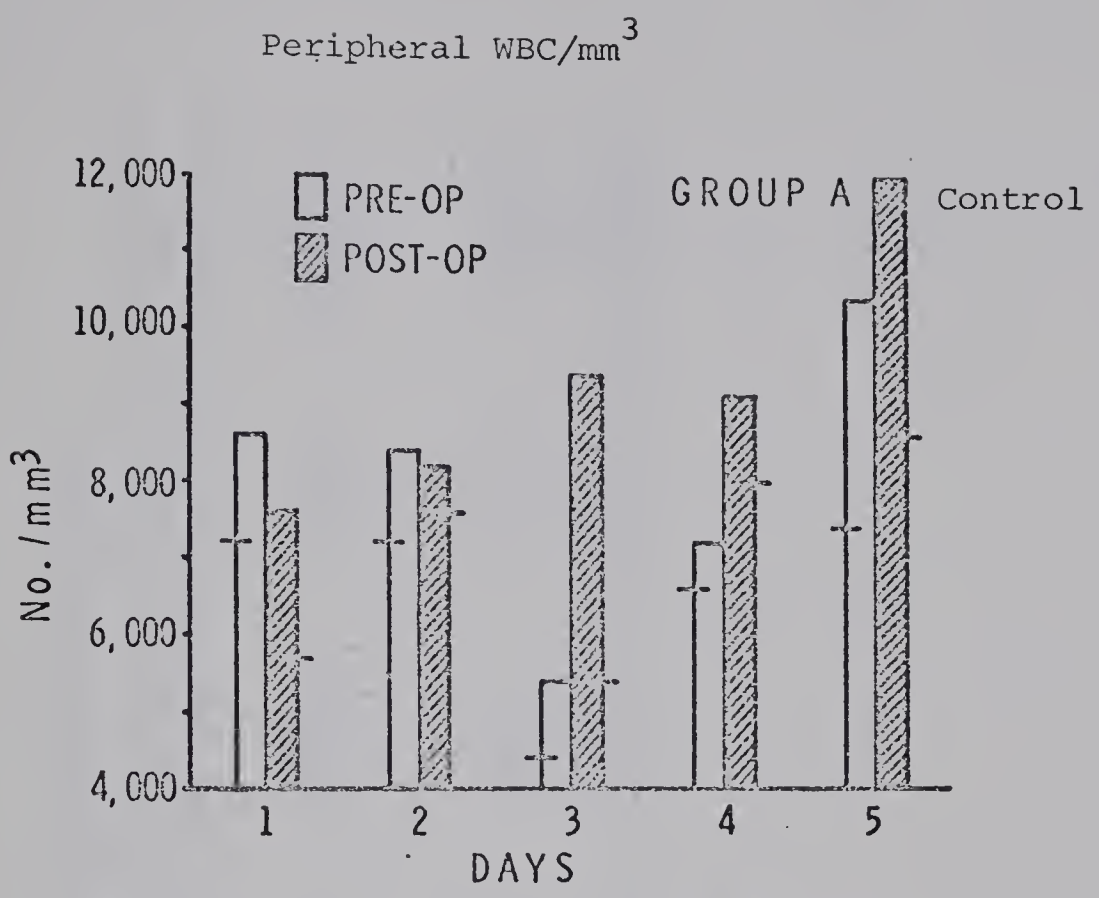
Counts of round cells and macrophages were not of particular note as can be seen from the tables and graphs of the respective cells (Tables 5 and 6, Figs. 5 and 6).

The number of fibroblasts in the control group rose gradually over the first 3 days and then steeply over days 4 and 5. The number

PERIPHERAL WHITE BLOOD CELLS/mm^3

Group A - Control	Day 1	Day 2	Day 3	Day 4	Day 5
Preoperative					
Number of rats	4	4	4	4	4
Mean	8625	8412.5	5412.5	7262.5	10,400
Std. Dev.	1389.469	1235.836	957.753	566.237	2986.078
Postoperative					
Mean	7625	8193	9425	9137.5	11,987.5
Std. Dev.	1890.546	604.226	4084.62	1101.797	3444.167
Diff. pre/post	-1000	-2195.5	+4012.5	+1875	+587.5
P value	<.2-.25	<.35-.4	<.05-.1	<.025-.05	--
Group B - Infected					
Preoperative					
Number of rats	4	4	4	4	4
Mean	6275	10,025	7050	7675	9262.5
Std. Dev.	673.918	1195.477	2970.690	1691.399	2076.605
Postoperative					
Mean	5787.5	9512.5	9900	11,812.5	13,862.5
Std. Dev.	1423.829	1110.086	3051.502	451.617	3367.584
Diff. pre/post	-487.5	-512.5	+2850	+4137	+4600
P value	<.25-.3	--	<.1-.15	--	--

Table 1


Peripheral WBC/mm3
12,000
10,000
8,000
6,000
4,000
No./mm3
PRE-OP
POST-OP
GROUP A
Control
1
2
3
4
5
DAYS

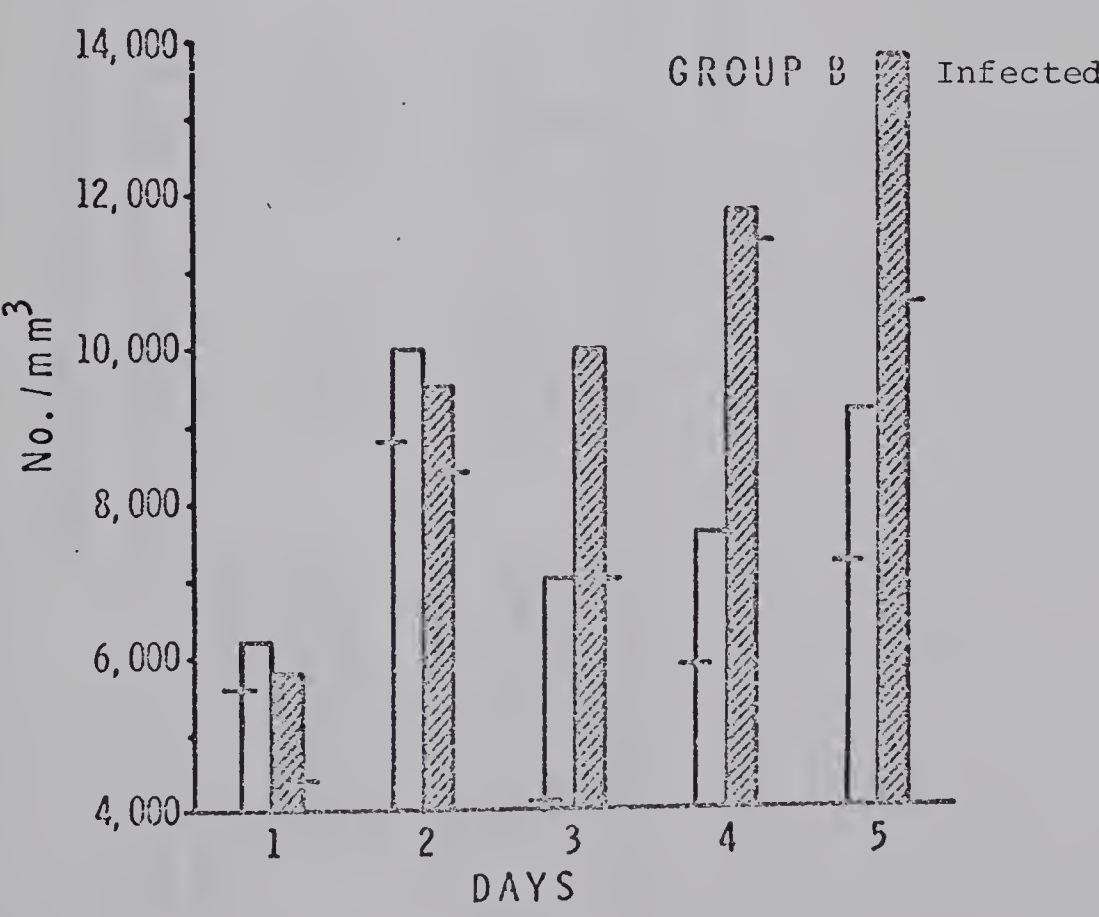

14,000
12,000
10,000
8,000
6,000
4,000
No./mm3
GROUP B
Infected
1
2
3
4
5
DAYS

Fig. 1

BONE MARROW - GRANULOCYTES AND PRECURSORS/200 CELLS

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Group A - Control					
Number of rats	4	4	4	4	4
Av. no. of cells	95±5.6	132±16.2	128±16.4	123±9.2	119.5±17.8
% no. of cells	47.5	66	64	61.5	59.7
E/M ratio	1:1	2:1	5:3	3:2	3:2
Group B - Infected					
Number of rats	4	4	4	4	4
Av. no. of cells	97.5±29.7	138±16.8	141±6.0	134.5±44	142±14.8
% no. of cells	48.7	69	70.5	67.2	71
P value	<.45	0.25.-3	<.1-0.15	<.3-.35	<.05-.1

Table 2

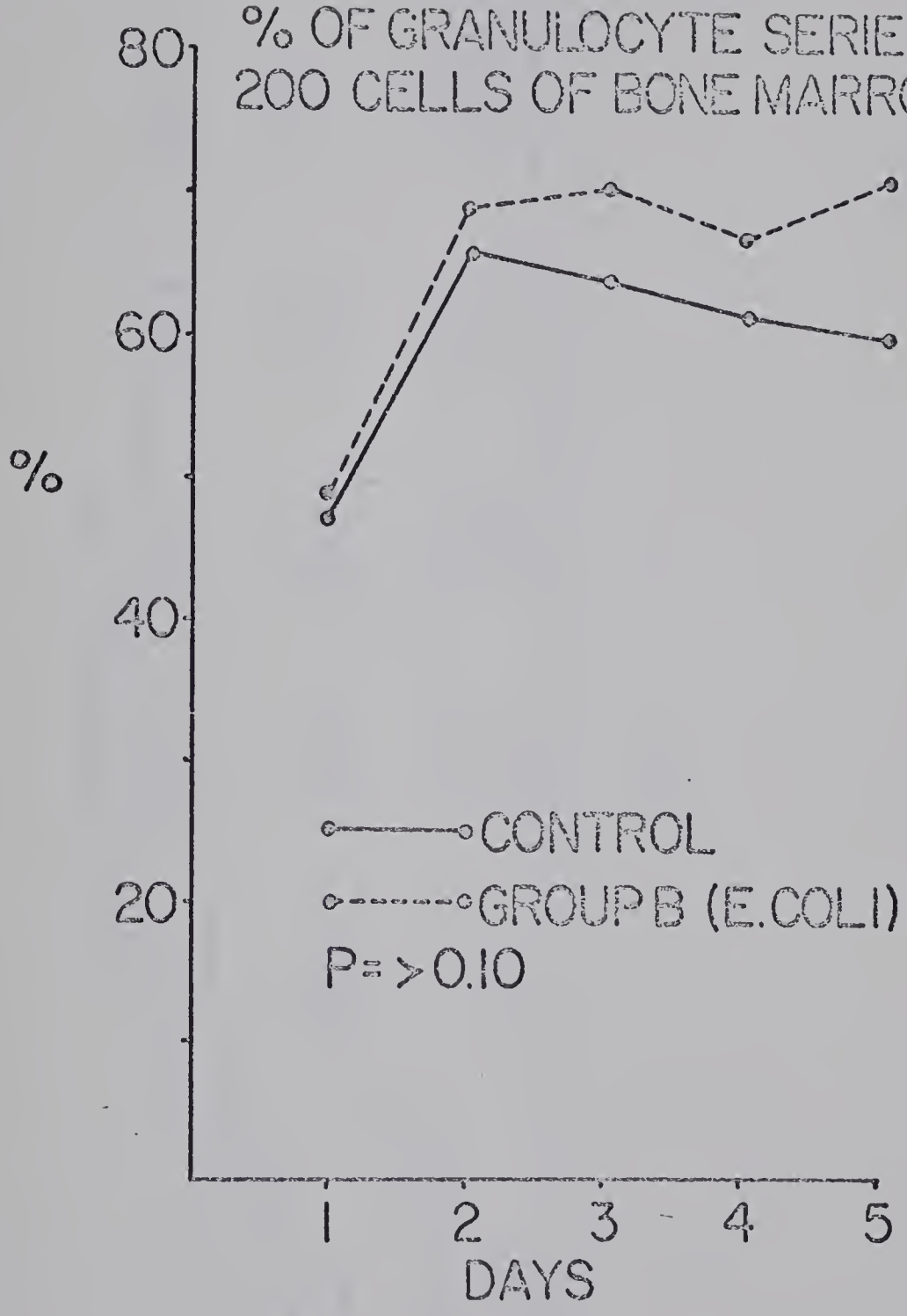

% OF GRANULOCYTE SERIES FOR
200 CELLS OF BONE MARROW
80
60
40
20
%
CONTROL
GROUP B (E.COLI)
P=>0.10
1
2
3
4
5
DAYS

Fig. 2

BURSTING PRESSURE IN cm. H_2O

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Group A - Control					
Number of rats	4	4	4	4	4
Mean	10.9	12	14.5	16.8	17.5
Std. Dev.	1.154	2.449	2.516	1.040	1.100
Group B - Infected					
Number of rats	4	4	4	4	4
Mean	5	6.5	9	13.5	13.2
Std. Dev.	1.632	4.041	3.915	0.577	1.258
P value	<.005-.01	<.025-.05	<.05-.1	<.005-.01	<.005-.01

Table 3

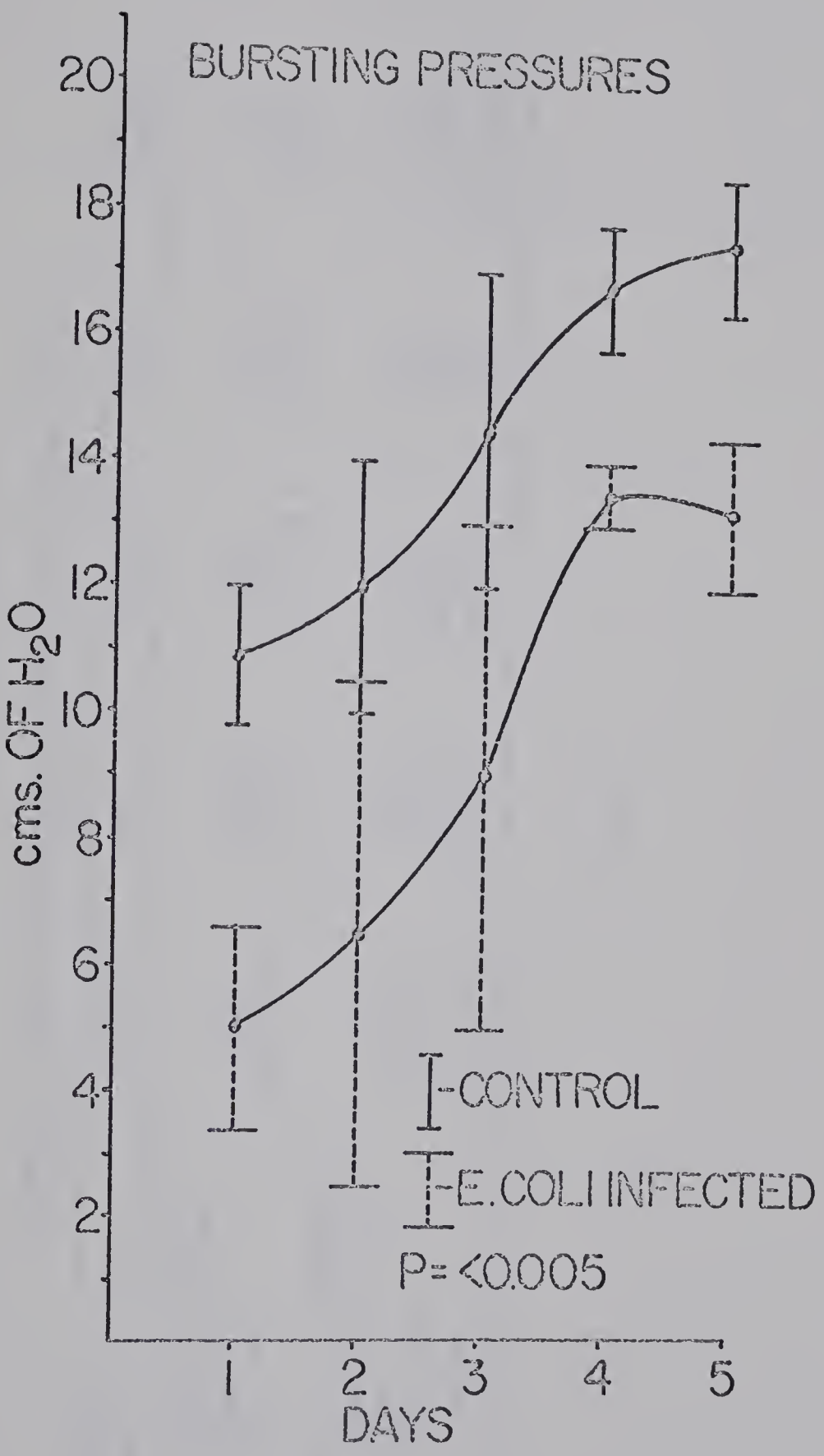

BURSTING PRESSURES
cms. OF H_2O
20
18
16
14
12
10
8
6
4
2
CONTROL
E. COLI INFECTED
P= <0.005
1
2
3
4
5
DAYS

Fig. 3

POLYMORPH COUNT AT ANASTOMOTIC SITE/UNIT VOLUME TISSUE

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Group A - Control					
Number of rats	4	4	4	4	4
Mean	665	472.5	290	151	130
Std. Dev.	119.482	21.189	157.598	31.219	42.142
Group B - Infected					
Number of rats	4	4	4	4	4
Mean	294.25	198	271	50	42
Std. Dev.	270.774	37.166	10.519	4.00	2.828
P value	<.025-.05	<.005	<.27-.137	<.005	<.01-.025

Table 4

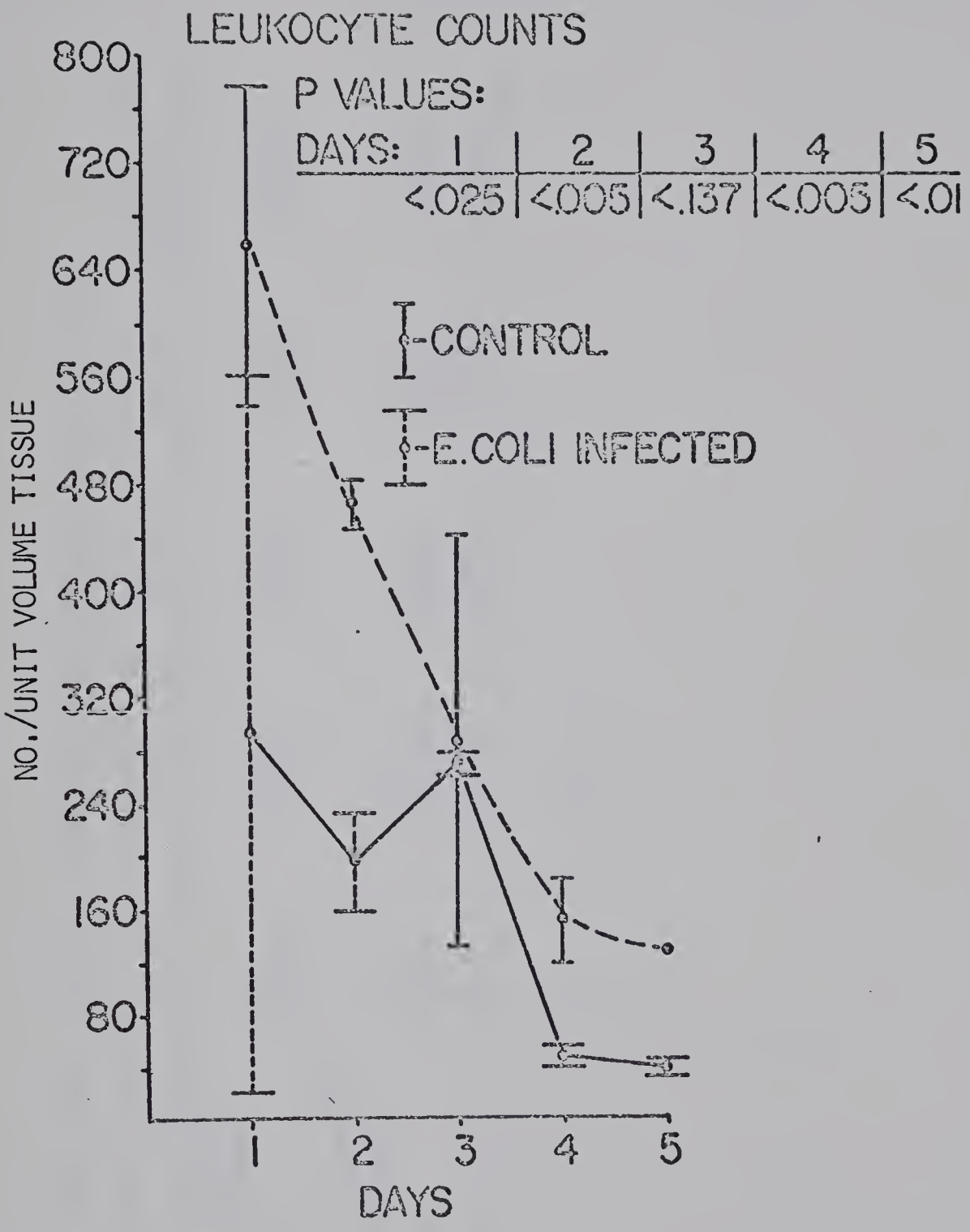

LEUKOCYTE COUNTS
P VALUES:
DAYS: 1 | 2 | 3 | 4 | 5
<.025 | <.005 | <.137 | <.003 | <.01
CONTROL
E.COLI INFECTED
NO./UNIT VOLUME TISSUE
800
720
640
560
480
400
320
240
160
80
1
2
3
4
5
DAYS

Fig. 4

ROUND CELL COUNT AT ANASTOMOTIC SITE/UNIT VOLUME TISSUE

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Group A - Control					
Number of rats	4	4	4	4	4
Mean	36	116	173	221	172
Std. Dev.	11.888	10.327	37.00	81.150	77.974
Group B - Infected					
Number of rats	4	4	4	4	4
Mean	77.5	103.5	164	178	149
Std. Dev.	50.474	33.00	24.657	16.492	11.489
P value	<0.2	<.25-0.3	<0.35	<.15-.2	<.3-.35

Table 5

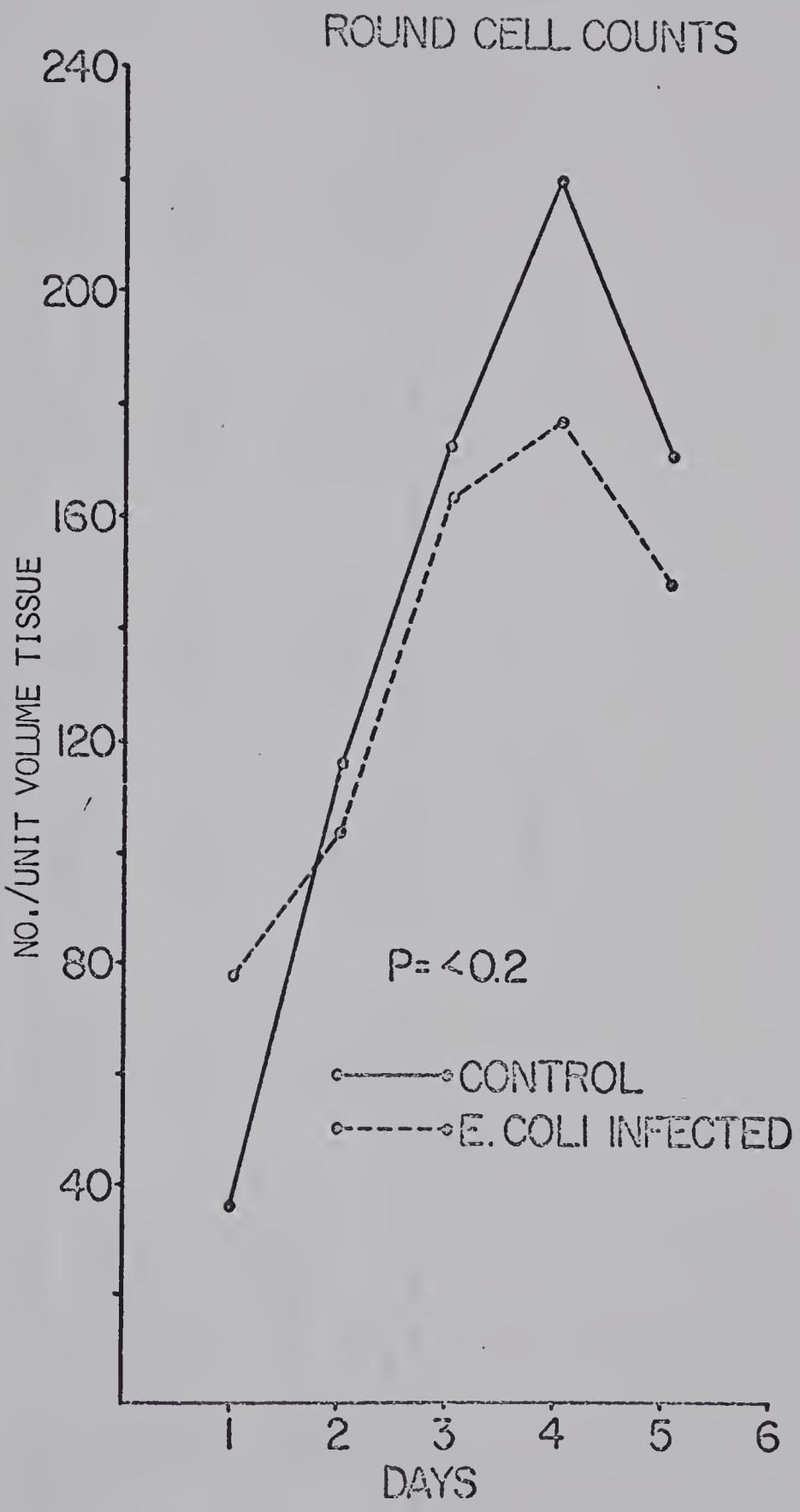

ROUND CELL COUNTS
240
200
160
120
80
40
NO./UNIT VOLUME TISSUE
P= <0.2
CONTROL
E. COLI INFECTED
1
2
3
4
5
6
DAYS

Fig. 5

MACROPHAGES/UNIT VOLUME TISSUE

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Group A - Control					
Number of rats	4	4	4	4	4
Mean	9	81	74.5	49	24
Std. Dev.	0	15.099	19.139	42.379	5.887
Group B - Infected					
Number of rats	4	4	4	4	4
Mean	22	92	81.5	25	24
Std. Dev.	0	29.574	39.979	13.316	11.313
P value	<.25-.3	<.25-.3	<.35-.4	<.02	<.45

Table 6

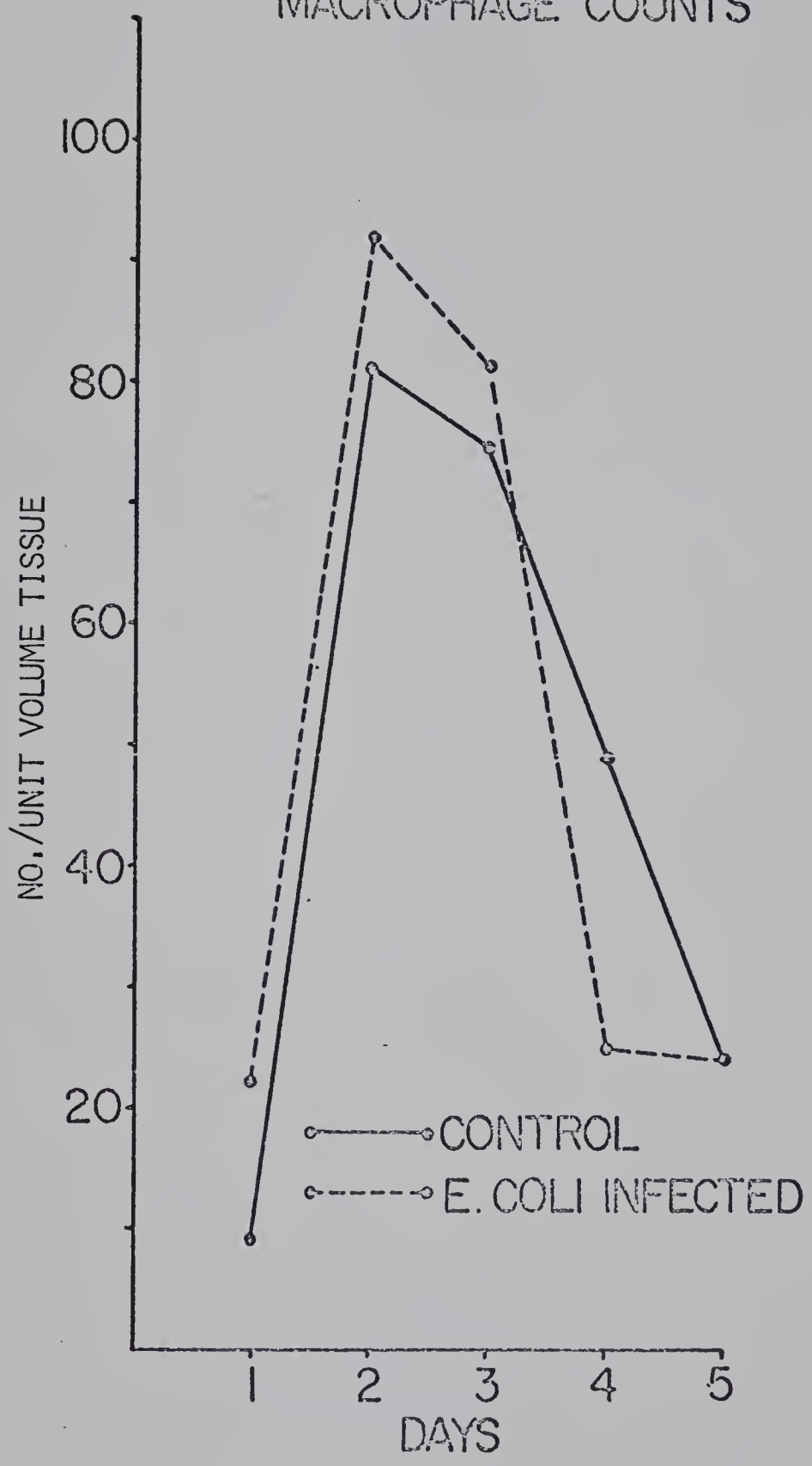

MACROPHAGE COUNTS
NO./UNIT VOLUME TISSUE
100
80
60
40
20
CONTROL
E. COLI INFECTED
1
2
3
4
5
DAYS

Fig. 6

of fibroblasts in the infected group was consistently lower than in the non-infected group except on the fourth day. However, these results statistically were not significant (Table 7, Fig. 7).

FIBROBLASTS/UNIT VOLUME TISSUE

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Group A - Control					
Number of rats	4	4	4	4	4
Mean	78	149	230	532	634
Std. Dev.	44.482	37.434	60.964	87.794	168.744
Group B - Infected					
Number of rats	4	4	4	4	4
Mean	31.5	87	173	646	443.2
Std. Dev.	12.922	14.00	73.855	331.034	136.762
P value	<.05-1.	<.025-.05	<.2-.25	<.25-.3	<.05-.1

Table 7

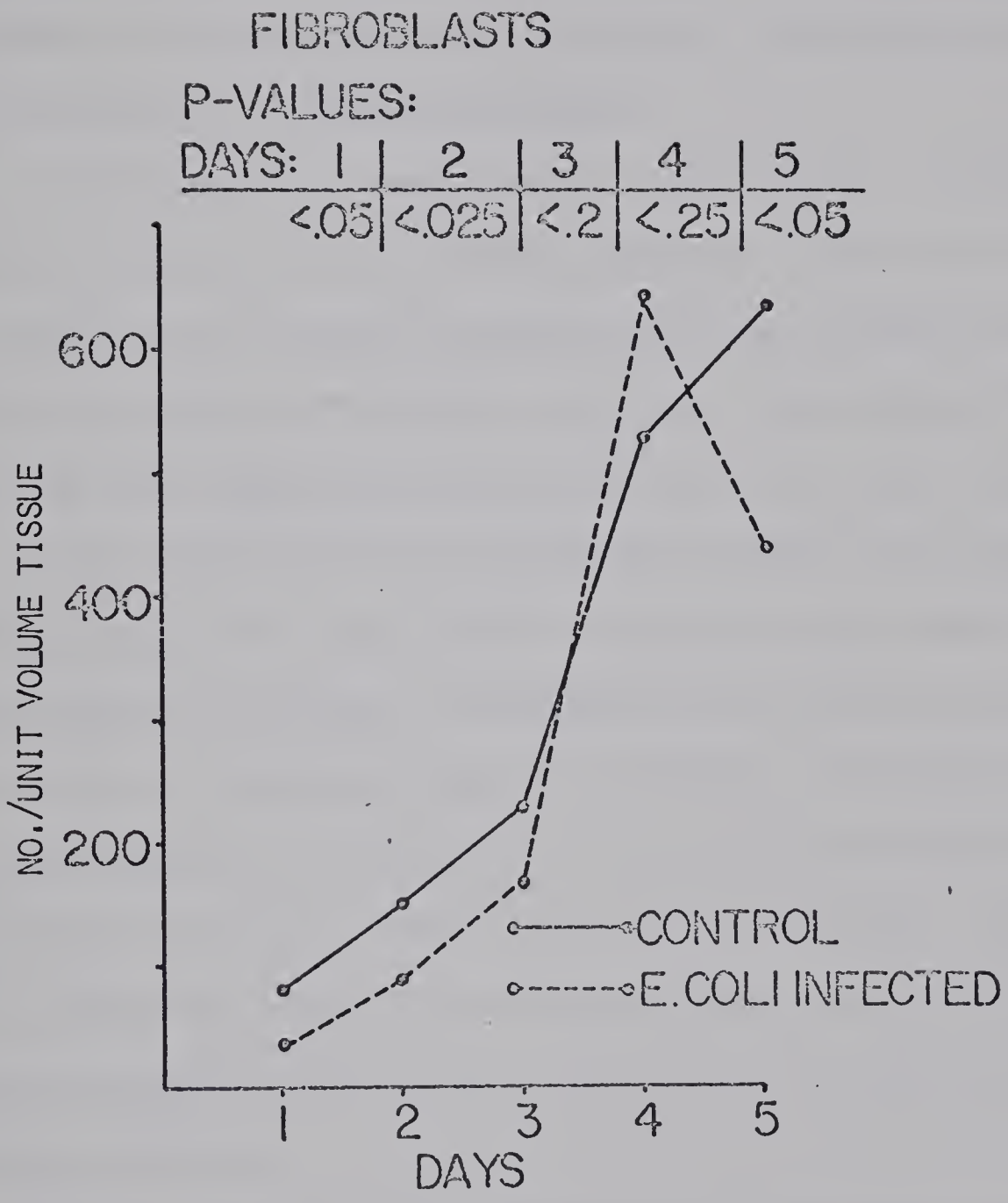

FIBROBLASTS
P-VALUES:
DAYS: 1 | 2 | 3 | 4 | 5
<.05 | <.025 | <.2 | <.25 | <.05
NO./UNIT VOLUME TISSUE
600
400
200
1
2
3
4
5
DAYS
CONTROL
E.COLI INFECTED

Fig. 7

DISCUSSION

This final part of this thesis will consist of an attempt to explain our persistent and significant experimental findings. Specifically, these findings are depressed neutrophil count at the site of the anastomosis in those animals infected with *E. coli* and a decreased bursting pressure in these same animals.

This discussion, then, will be in four parts. I shall first consider the probable cause for these findings. This will be followed by a discussion of the many possible mechanisms of action which might explain the results. In the last two parts I will present some hypotheses and some suggestions for the direction of further study.

Since the significant experimental results are confined to the infected group, it is only logical to assume that the cause is related to the infecting organism. As mentioned in an earlier section, *E. coli* antigens may be flagellar, capsular or somatic. The somatic antigen of the gram negative enterobacteria is a complex β-lipopolysaccharide which makes up the cell wall. This has been termed endotoxin. This was done to distinguish the bacterial toxin released upon death and lysis of gram negative organisms from the exotoxins secreted by living, usually gram positive, bacteria.

Following an extensive search of the literature and consultations with bacteriologists we are unable to find reports of an antigen other than the somatic O antigen which would explain our findings. There is evidence which enables us to hypothesize *E. coli* endotoxin as the cause of these findings.

The possibility of an abnormal metabolite of *E. coli* causing a

local depression of neutrophils, however, cannot be ruled out completely. This is especially true in view of the relatively small dose of bacteria which we used. Some strains of *E. coli* have been reported to produce fibrinolytic enzymes and also a necrotizing factor. It is, therefore, possible that the fibrin might be directly attacked and the neutrophils damaged by bacterial products (Smith, Conant *et al.*, 1960).

Gram negative endotoxins have been extensively studied over the past fifteen years by a large number of workers in disciplines ranging from clinical surgery to experimental immunology. Their interest has been mainly to determine the pathophysiology and from this the management of the shock state which may arise from severe bacteraemias and septicaemias of gram negative origin. The studies have been usually carried out with a purified β-lipopolysaccharide endotoxin derived either from *Pseudomonas aerugenosa* or *E. coli*. We have been unable to find any studies whatever which show a decreased number of polymorphs at the site of infected tissue. In fact, we have been unable to find any studies similar to ours — that is, situations where cells were actually counted at a point known to be infected.

There are, however, several studies on the effect of intraperitoneal injections of endotoxin, especially on the virulence of associated infections. In some of these studies white blood counts were done on both circulating blood and peritoneal fluid.

There are several reviews (Rosen, 1961; Braude, 1964) which discuss the major pathological effects of gram negative endotoxins. Many varieties of experimental animal have been used, most notably rats, mice, rabbits and dogs. Mulholland and Cluff (1964) found when they injected a small quantity of bacterial lipopolysaccharide they obtained

a transient leukopenia in the peripheral blood. This leukopenia lasted only six hours, by which time the peripheral white count had returned to its level at time 0. In our present work we did no experimental leukocyte counts prior to 24 hours beyond time 0. This transient leukopenia is a well-recognized phenomenon following intravenous injection of endotoxin.

A second phenomenon associated with endotoxin administration is the appearance of a fever between fifteen and thirty minutes following the injection of endotoxin (Rosen, 1961). This fever spike, which is frequently accompanied by a chill and a rigor, usually begins to abate after an hour. If the dose of endotoxin has been small the fever will not return. If a larger dose of pyrogen is injected, the first fever is followed in a few hours by a second fever. Braude (1964) claims that the initial fever is the result of heat retention from a decreased skin circulation. The secondary fever is believed due to the release of pyrogens from injured leukocytes. Another opinion holds that bacterial lipopolysaccharide may directly stimulate the hypothalmic thermoregulatory centres.

In addition to causing a fever and transient leukopenia, bacterial endotoxins have the property of being able to initiate a shock syndrome. This property has been under study by many groups in many different places and the body of literature which has accumulated in the past ten years is immense. A large amount of the research which has been carried out has been related to the prevention and treatment of septic shock as it relates to patients. At the same time much effort has been expended in trying to determine the pathophysiology of septic shock. Many difficulties have arisen mainly in transferring the results of animal experi-

ments to human patients. While of great interest to the clinician, this subject goes far beyond the bounds of this paper and will not be discussed.

It is, then, my thesis that there is a direct relation between the decreased bursting strength of the infected anastomoses and the relative local neutropenia. The reason that infected wounds are weaker is that there is less fibrin and collagen in them and this is a result of there being a poorer inflammatory response present to clean up the debris and allow for the synthesis of mucopolysaccharides. It may also be due in part to the fibrinolysins previously mentioned.

The infecting organisms are the cause of the neutropenia. They have been shown, or rather endotoxin has been shown, to depress the peripheral leukocyte count for up to six hours following its injection (Mulholland and Cluff). Our work has shown a significant decrease in the level of neutrophils at the site of a bowel anastomosis infected with *E. coli*. There are several explanations to account for these findings.

Endotoxin may depress the maturation and/or release of neutrophils from the bone marrow transiently. This would explain both findings. Unfortunately there is no support for this concept in the literature.

Endotoxin may suppress the margination and emigration of neutrophils from the circulation. One of the effects of endotoxin not discussed earlier is the phenomenon of intravascular clotting which occurs in capillaries and small blood vessels in the animal in septic shock. This would account for the local findings, but would be reflected in an elevated rather than depressed peripheral white count. Furthermore, our rats were not in shock. In fact, they were minimally ill.

It is conceivable that endotoxin could interfere with chemotaxis

but there seems to be some doubt as to the true nature of chemotaxis.

Local destruction of neutrophils at the site of infection is the final possibility. There is a lack of supportive evidence, but together with bone marrow suppression it seems to me possible. Endotoxin is known to render lysosomal membrane less stable and it is therefore conceivable that neutrophils arriving at an infected anastomosis might, in the course of engulfing the organism, release its β-lipopolysaccharide cell wall which in turn would cause the remaining lysosomes to rupture with intracellular release of enzymes and consequent autolysis and cell destruction.

This is, of course, pure hypothesis but with some support in other quarters (Schumer and Nyhus, 1969). Furthermore, this work lends itself to more study in certain fields and raises some questions.

Do the infecting organisms multiply and provide a form of "sustained release" not seen in other experiments?

Do the local conditions permit or cause the organisms to form some other long- or short-chain fatty acids which may have a hitherto unknown effect on neutrophils?

Hydrocortisone has a wide variety of effects in the mammal. Its effects on wound healing have been discussed and its effects on bone marrow are well known. Recently it has been shown to have a stabilizing effect on lysosomal membranes. It would be interesting to see what, if any, effect hydrocortisone has on the number of neutrophils at the site of infected and uninfected anastomoses.

In view of the work of Mulholland and Cluff, further work will have to involve sacrificing rats hourly after time 0 up to 12 hours, then 18 hours and finally 24 hours. This is an area on which, regret-

tably, our work has shed no light at all.

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